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Molecular annotation of type specimens of *Russula* species described by W.A. Murrill from the southeast United States

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Abstract — Twenty-five historical type collections of North American *Russula* species described by W.A. Murrill were sequenced and analyzed for placement in a phylogenetic and taxonomic framework. Molecular data was successfully obtained from sixteen type specimens (66%), of which eight species have never before been placed in an infrageneric classification system. Classifications and synonymization of taxa proposed by Rolf Singer and others are evaluated. *Russula albimarginata* and *R. emeticiformis* are suggested as synonyms of *R. vinacea,* a widespread and common species in the eastern United States. Four taxa of unknown classification (*R. albimarginata, R. pinophila, R. subcremeiceps, R. subrubescens*) have been classified to subsection level, and an alternative classification is proposed for *R. westii*. A morphological comparison of a collection from British Columbia (Canada) with 99% sequence similarity to *R. levyana* in the Internal Transcribed Spacer 1 (ITS1) region indicates that this region alone may be inadequate for species barcoding in *Russula*.

Key words —phylogenetics, TENN herbarium, high performance DNA extraction, Florida

Introduction

Between 1938 and 1948, W.A. Murrill described 110 species in the genus *Russula* Pers. almost exclusively from the area surrounding Gainesville, Florida (Mycobank 2014). Of the 334 *Russula* species described from the United States, Murrill's work comprises the largest number described by a single mycologist (Buyck 2007). Unfortunately, most of the taxa are poorly known, due partly to Murrill's short diagnoses that focused on macromorphological characters and partly to the lack of extensive studies of *Russula* in the southeast U.S. One notable exception to the lack of attention given to *Russula* in the region is a series of type studies by Hesler (1960, 1961). These studies treat 191 *Russula* species, including 87 species described by Murrill, and they contribute data from microscopic descriptions of the pileus cuticle, lamellar cystidia, and

spore morphology with some discussion of taxonomic affinities proposed by Rolf Singer. Hesler received authentic and type material from major herbaria (FH, MICH, NY, NYS, and FLAS) for these studies, and in the case of Murrill's collections, many were sent as isotypes to be accessioned at TENN (herbarium abbreviations per Thiers 2013).

Central to our current understanding of *Russula* in North America are the studies of Rolf Singer (Singer 1986), which include the most comprehensive infrageneric classification system to include North American taxa. Singer treated a number of Murrill's taxa in various editions of "The *Agaricales* in Modern Taxonomy" (Singer 1951, 1975, 1986), and it is my objective to test these proposed relationships in a phylogenetic context. A recent initiative to revive the taxonomy of North American *Russula* has led to a number of published type studies using advanced modern microscopy and morphometrics and unpublished phylogenies upon which a number of taxonomic affinities have been proposed (Adamčík & Buyck 2010, Adamčík & Buyck 2011a, Adamčík & Buyck 2011b, Adamčík et al. 2013, Buyck & Adamčík 2011a, Buyck & Adamčík 2011b, Buyck & Adamčík 2013). With these renewed concepts, new reports of historical *Russula* taxa are emerging (Adamčík et al. 2010, Buyck et al. 2008, Buyck et al. 2011). The only other comprehensive treatment of a majority of Murrill's taxa can be found in the appendix of Kibby & Fatto (1990), who coded features of southern species for use in their synoptic key system, which also included abbreviated species descriptions. The objective of this study is to contribute to the revival of *Russula* taxonomy in North America by determining whether Murrill's type material at TENN can be molecularly annotated and by exploring what sort of taxonomic inferences can be drawn from these data.

Molecular methods

Following an inventory of *Russula* type material deposited at TENN, 25 type collections of species designated by W.A. Murrill were selected for DNA extraction and molecular annotation (Table 1). In most cases, it was determined that ample material was available to allow using 50 mg of dried material for DNA extraction. The sporocarp material and a pinch of sterile sand were placed in a mortar to which liquid nitrogen was added. The frozen material was ground to a fine powder with a pestle and scraped into a 1.5 mL microtube with a metal spatula. DNA extraction protocols followed the E.Z.N.A. HP Fungal DNA Kit (Omega Bio-Tek, Norcross, Georgia) with the following notable exceptions to improve end-product DNA concentration: 1) 10 µL 2-mercaptoethanol was added to the samples in buffer, and they were allowed to incubate at 65°C for 24 hours (vortexing at the beginning of incubation and intermittently during the final 30 minutes). 2) After the prescribed 300 µl of supernatant and associated reagents were centrifuged through the HiBind DNA column, the remaining supernatant was run through a second round of centrifugation. 3) Two rounds of DNA elution using 50 µL of Elution buffer were performed after a 5 minute incubation at 65°C with the buffer added. Dilutions of 1:10 were made from the genomic DNA product.

TABLE 1. Sequence data from *Russula* taxa selected for phylogenetic comparison with Singer's infrageneric classification

COLLECTIONS[1]	TYPE DESIG.	COLL. #	TENN #	ITS1	ITS2	GENBANK MATCH	INFRA. CLASS.	PHYLO. CLASS.	GENBANK #
R. westii	Isotype	F16404	21262	✓	✓	93% *R. aeruginea* CAN	*Lilaceinae*	*Subcompactinae*	KF810121
R. brunneipes	Isotype	F19537	21229	✓		98% *R.* sp. MA	unknown	uncertain	KF810122
R. subrubescens	Paratype	F18349	21254	✓		97% orchid root tip OH	unknown	*Urentes*	KF810123
R. variicolor	Isotype	F9513	21259	✓		99% Uncultured Eur	*Amoeninae*	*Amoeninae*	KF810124
R. subcremeiceps	Isotype	F38920	21230	✓		98% *R.* sp. VT	unknown	*Chamaeleontinae*	KF810125
R. alachuana	Isotype	F9510	21221	✓		99% orchid root tip MEX	*Amoeninae*	*Amoeninae*	KF810126
R. albimarginata	Isotype	F19447	21225		✓	99% *R. atropurpurea* TN	unknown	subsect. *Russula*	KF810127
R. levyana	Isotype	F15859	21235	✓		99% *R. xerampelina* BC	*Xerampelinae*	*Xerampelinae*	KF810128
R. vinosirosea	Isotype	F18677	21261	✓		94% *R.* sp. BC	unknown	uncertain	KF810129
R. mutabilis	Isotype	F17943	21237	✓		98% orchid root tip THA	*Subvelatae*	*Ingratae*	KF810130
R. fragiloides	Isotype	F18001	21232	✓		90% *R. raoultii* Eur	subsect. *Russula*	subsect. *Russula*	KF810131
R. australirosea	Isotype	F38859	21228	✓		92% *R.* sp. CA	*Lilaceinae*	uncertain	KF810132
R. emeticiformis	Isotype	F9535	21231	✓		96% *R. atropurpurea* TN	subsect. *Russula*	subsect. *Russula*	KF810133
R. pinophila	Isotype	F17982	21240	✓		96% *R. xerampelina* BC	unknown	*Xerampelinae*	KF810134
R. subsulphurea	Paratype	F18743	21255	✓	✓	100% environ NC	unknown	uncertain	KF810135
R. subrubescens	Isotype	F18339	21253	✓	✓	99% Uncultured MEX	unknown	*Urentes*	KF810136
R. pervirginea Murrill	Isotype	F17266	21238			FAILED			
R. subflava Murrill	Isotype	F16256	21249			FAILED			
R. venusta Murrill	Isotype	F17836	21260			FAILED			
R. subglauca Murrill	Isotype	F17781	21250			FAILED			
R. alutaceiformis Murrill	Isotype	F17703	21226			FAILED			
R. testaceiceps Murrill	Isotype	F15916	21257			FAILED			
R. subobscura Murrill	Isotype	F18301	21251			FAILED			
R. patriotica Murrill	Isotype	F18066	21258			FAILED			
R. subpusilla Murrill	Isotype	F9512	21252			FAILED			
R. mutabilis	none	DPL10654		✓	✓	N/A	*Subvelatae*	*Ingratae*	KF810137
R. cf. *aquosa* Leclair	none	BPL271		✓	✓	N/A	subsect. *Russula*		KF810138
R. vinacea	none	BPL257		✓	✓	N/A	subsect. *Russula*		KF810139

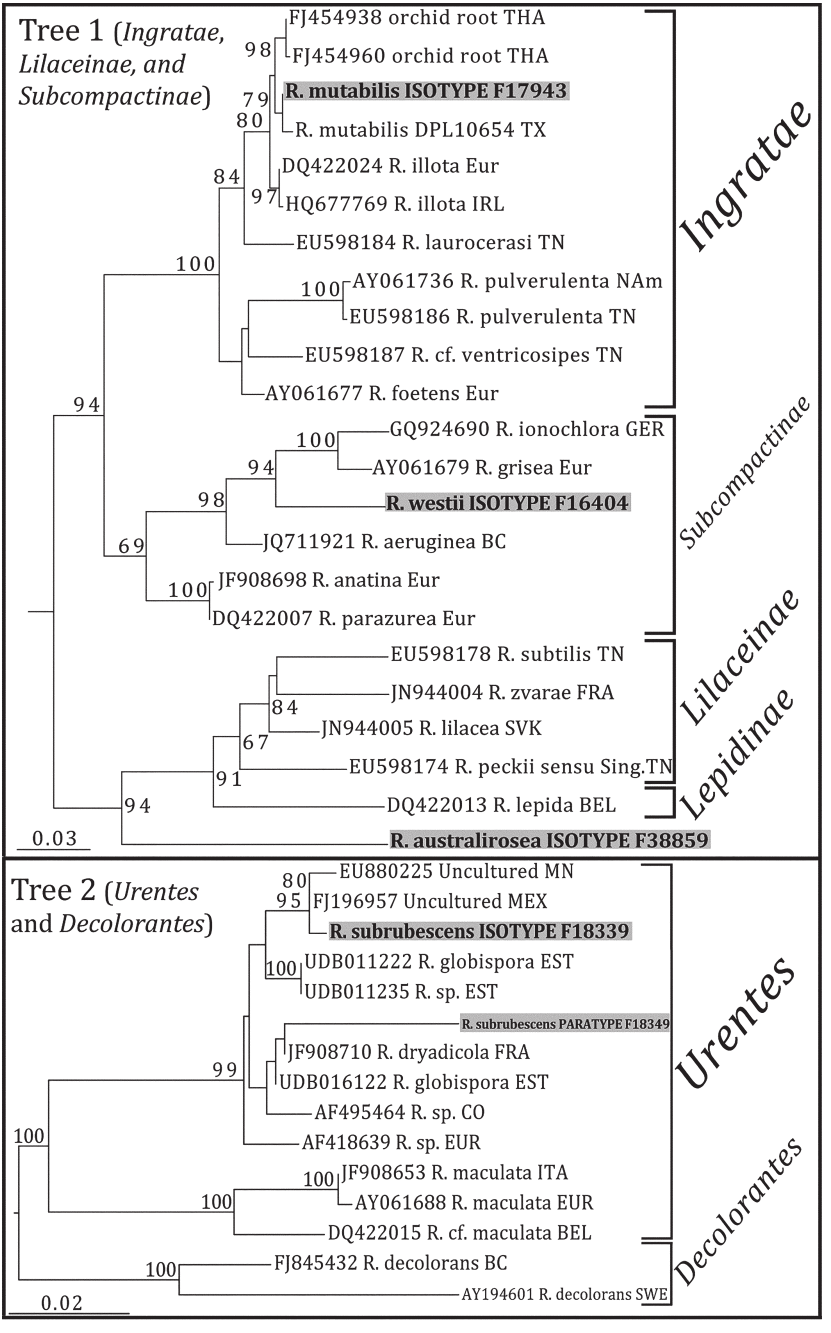
[1]Type collections accessioned at TENN not sampled (out on loan) were: *Russula albiduliformis* Murrill, *R. heterosporoides* Murrill, *R. lutescentifolia* Murrill, *R. pseudofoetens* Murrill, *R. rooseveltiana* Murrill, *R. subalbidula* Murrill, *R. subbrunneipes* Murrill, and *R. subgraminicolor* Murrill.

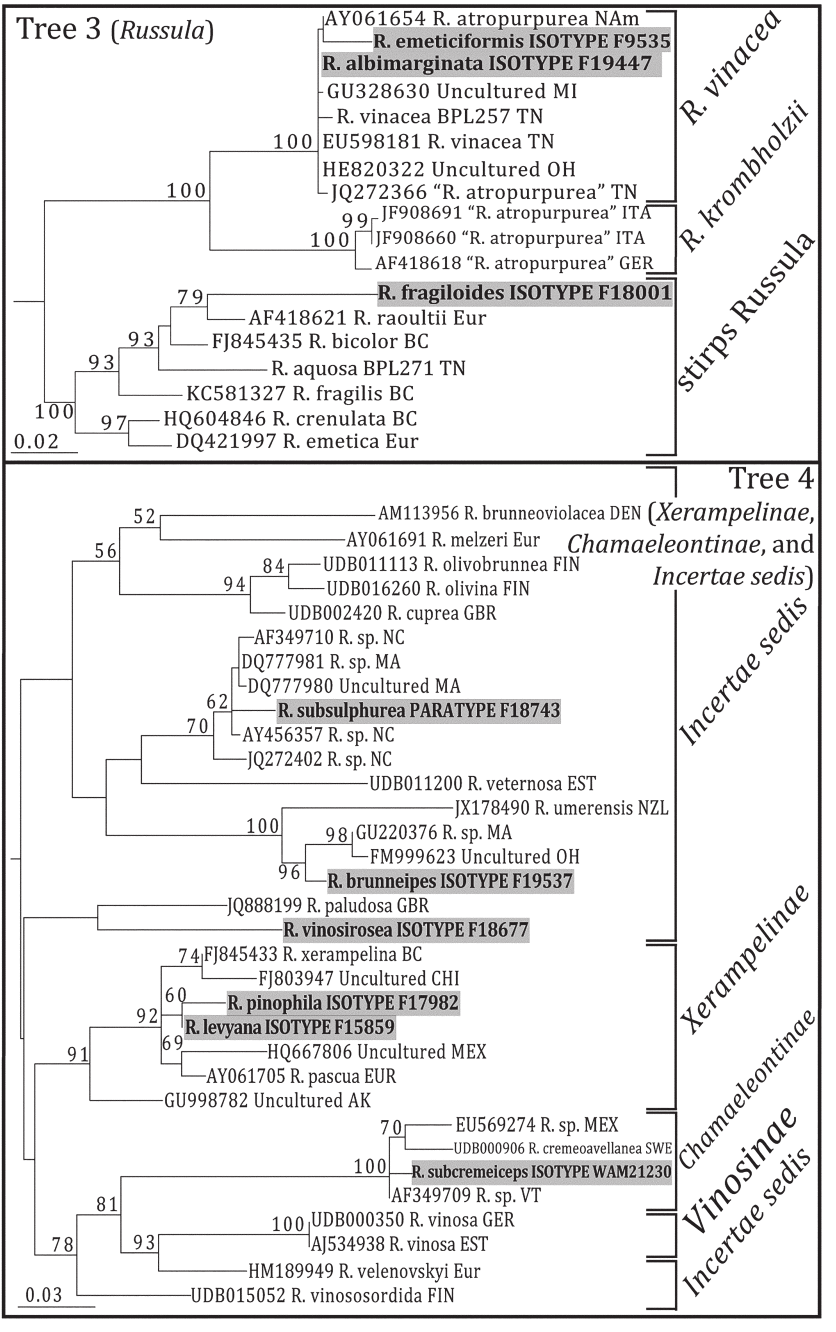
Initially, products were screened using the primer pair ITS1F-ITS4 (Gardes & Bruns 1993, White et al. 1990). PCR amplification protocols and controls used the requisite reagents of sterile water, 5× GoTaq buffer (Promega, Madison, Wisconsin), GoTaq, and 10 mM solution of dNTPs (Invitrogen Corp., Carlsbad, California). The DNA concentration in master mix solution was increased from 1:24 to 4:21 for improved PCR product concentration, and the samples were run using an ITS protocol on a Bio-Rad C1000 thermal cycler (Bio-Rad, Hercules, California). PCR product quality was visualized using gel electrophoresis on a 1% agarose gel prepared with ethidium bromide and then transilluminated using UV light. Specimens for which no banding was present were re-screened using the primer pairs ITS1F-ITS2 and 5.8SR-ITS4. Specimens for which a band was produced were cleaned with a QIAquick PCR purification kit (QIAGEN, Valencia, California). Sequence reactions were prepared and purified following Birkebak et al. (2013). Purified samples were sent to the Molecular Biology Resource Facility at U.T. to be sequenced using a 3730 DNA analyzer (Applied Biosystems, Grand Island, NY).

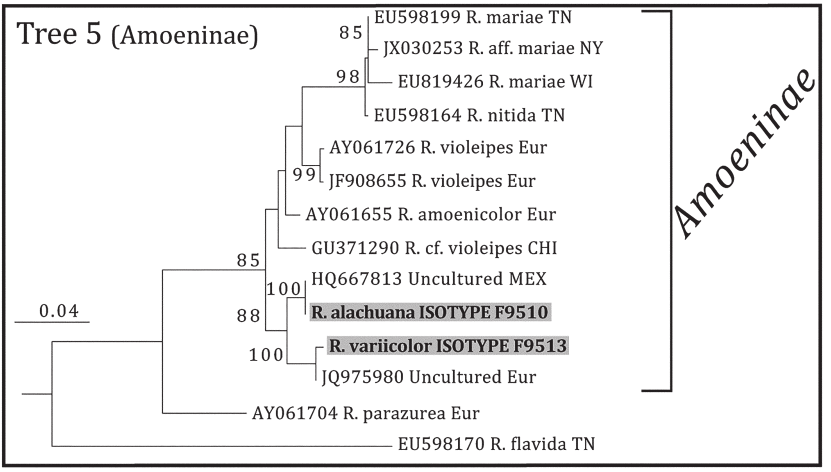
Sequences were assembled using Sequencher 4.9 (Gene Codes Corp., Ann Arbor, Michigan). For types where ITS1 and ITS2 sequences were produced separately, a bridge of repeated N's was used to form a complete ITS sequence. Using the BLAST program (Altschul et al. 1990) sequences were queried against the GenBank database (NCBI, Bethesda, Maryland) and visualized using the distance tree of results. Sequences that blasted with 98% similarity or higher were selected for phylogenetic analysis as candidate conspecifics or closely related taxa as well as identified sequences with at least 90% similarity for placement in an infrageneric group. Additional vouchered sequences of nomenclatural type species or representatives of infrageneric groups based on morphological synapomorphies were selected for testing infrageneric hypotheses. Alignments were formed using MAFFT version 7 (Katoh & Standley 2013) using default options and then adjusted manually in MacClade 4.08 (Maddison & Maddison 2005). Phylip file formats were created in Seaview 4.4.2 (Gouy et al. 2010). Phylogenies were reconstructed using maximum likelihood (ML) in raxmlGUI 1.2 (Silvestro & Michalak 2012, Stamatakis et al. 2008) with 1000 bootstrap replicates. Bootstrap values 70% and above are considered good support for clades. State/provincial abbreviations for the U.S. and Canada are according to national postal codes and country abbreviations use the three-letter ISO code (International Organization for Standardization, Geneva, Switzerland). All sequences are deposited in GenBank.

Molecular results

Sequence data were recovered from sixteen of twenty-five type specimens (64% screened) with collection dates ranging from 1932 to 1944 (Table 1). Only *R. westii* Murrill (KF810121; 4% screened) was recovered as a full ITS sequence using the ITS1F-ITS4 primer pair. When the spacer regions were amplified separately, the ITS1 region was successfully amplified in fourteen of fifteen instances (93%) using the ITS1F-ITS2 primer pair. The ITS2 region was successfully amplified for three out of fifteen types (20%) using the 5.8SR-ITS4 primer pair. Both ITS1 and ITS2 were produced for *R. subrubescens* Murrill


Tree 1 (*Ingratae, Lilaceinae, and Subcompactinae*)
FJ454938 orchid root THA
FJ454960 orchid root THA
R. mutabilis ISOTYPE F17943
R. mutabilis DPL10654 TX
DQ422024 R. illota Eur
HQ677769 R. illota IRL
EU598184 R. laurocerasi TN
AY061736 R. pulverulenta NAm
EU598186 R. pulverulenta TN
EU598187 R. cf. ventricosipes TN
AY061677 R. foetens Eur
GQ924690 R. ionochlora GER
AY061679 R. grisea Eur
R. westii ISOTYPE F16404
JQ711921 R. aeruginea BC
JF908698 R. anatina Eur
DQ422007 R. parazurea Eur
EU598178 R. subtilis TN
JN944004 R. zvarae FRA
JN944005 R. lilacea SVK
EU598174 R. peckii sensu Sing.TN
DQ422013 R. lepida BEL
R. australirosea ISOTYPE F38859
0.03
Ingratae
Subcompactinae
Lilaceinae
Lepidinae
Tree 2 (*Urentes* and *Decolorantes*)
EU880225 Uncultured MN
FJ196957 Uncultured MEX
R. subrubescens ISOTYPE F18339
UDB011222 R. globispora EST
UDB011235 R. sp. EST
R. subrubescens PARATYPE F18349
JF908710 R. dryadicola FRA
UDB016122 R. globispora EST
AF495464 R. sp. CO
AF418639 R. sp. EUR
JF908653 R. maculata ITA
AY061688 R. maculata EUR
DQ422015 R. cf. maculata BEL
FJ845432 R. decolorans BC
AY194601 R. decolorans SWE
0.02
Urentes
Decolorantes


Tree 3 (Russula)
AY061654 R. atropurpurea NAm
R. emeticiformis ISOTYPE F9535
R. albimarginata ISOTYPE F19447
GU328630 Uncultured MI
R. vinacea BPL257 TN
100
EU598181 R. vinacea TN
HE820322 Uncultured OH
100
JQ272366 "R. atropurpurea" TN
99
JF908691 "R. atropurpurea" ITA
100
JF908660 "R. atropurpurea" ITA
AF418618 "R. atropurpurea" GER
R. fragiloides ISOTYPE F18001
79
AF418621 R. raoultii Eur
93
FJ845435 R. bicolor BC
93
R. aquosa BPL271 TN
KC581327 R. fragilis BC
100
97
HQ604846 R. crenulata BC
0.02
DQ421997 R. emetica Eur
R. vinacea
R. krombholzii
stirps Russula
Tree 4 (Xerampelinae, Chamaeleontinae, and Incertae sedis)
52
AM113956 R. brunneoviolacea DEN
AY061691 R. melzeri Eur
56
84
UDB011113 R. olivobrunnea FIN
94
UDB016260 R. olivina FIN
UDB002420 R. cuprea GBR
AF349710 R. sp. NC
DQ777981 R. sp. MA
DQ777980 Uncultured MA
62
R. subsulphurea PARATYPE F18743
70
AY456357 R. sp. NC
JQ272402 R. sp. NC
UDB011200 R. veternosa EST
JX178490 R. umerensis NZL
100
98
GU220376 R. sp. MA
FM999623 Uncultured OH
96
R. brunneipes ISOTYPE F19537
JQ888199 R. paludosa GBR
R. vinosirosea ISOTYPE F18677
74
FJ845433 R. xerampelina BC
FJ803947 Uncultured CHI
60
R. pinophila ISOTYPE F17982
92
R. levyana ISOTYPE F15859
69
91
HQ667806 Uncultured MEX
AY061705 R. pascua EUR
GU998782 Uncultured AK
70
EU569274 R. sp. MEX
UDB000906 R. cremeoavellanea SWE
100
R. subcremeiceps ISOTYPE WAM21230
AF349709 R. sp. VT
81
100
UDB000350 R. vinosa GER
78
93
AJ534938 R. vinosa EST
HM189949 R. velenovskyi Eur
0.03
UDB015052 R. vinososordida FIN
Incertae sedis
Xerampelinae
Chamaeleontinae
Vinosinae
Incertae sedis



Figures 1–3. Unrooted phylogenies derived from maximum likelihood analysis of complete or partial ITS rDNA sequences of types of *Russula* taxa described by W.A. Murrill with included publicly accessioned sequences for phylogenetic classification. Type sequences are highlighted and bolded. Publicly accessioned sequences are included if they share a 98% sequence similarity to a type or are identified to species and within 90% sequence similarity.

(KF810136) and *R. subsulphurea* Murrill (KF810135). Five phylogenetic trees were constructed by utilizing nomenclatural type species or representatives of infrageneric groups based on morphological synapomorphies to test proposed infrageneric classifications and synonymizations of Murrill's taxa. Monophyletic clades with high bootstrap support that include type species or representatives of known infrageneric classification were used to define infrageneric groups; however, relationships between these clades should not be considered significant due to the lack of gene and taxon sampling.

To test whether the ITS1 region can consistently and accurately separate species in *Russula,* I examined collection FJ845433 from British Columbia, Canada, which shares a 99% sequence similarity with 309/310 sites of the ITS1 region from the isotype of *R. levyana* Murrill. A disjunct or continuous range of over 4000 km across continental North America for one *Russula* species would be surprising, especially considering the significant host, habitat, and climate differences. Although both specimens certainly belong in *R.* subsect. *Xerampelinae* Singer, in the pileus of the Canadian collection, I found large spores, predominantly olive green tones, and no long, attenuated hyphal terminations in the suprapellis — features inconsistent with modern examinations of the type and recent collections of *R. levyana* and which support separation based on a morphological species concept (Adamčík & Buyck 2010).

Tree 1 (*Ingratae*, *Lilaceinae*, and *Subcompactinae*) **Figure 1**

The isotype of *R. mutabilis* Murrill shares a 100% sequence similarity with a recent specimen collected in Texas determined as *R. mutabilis* (KF810137). The species is in a well-supported clade that includes sequences identified as *R. illota* Romagn. and environmental sequences of an orchid associate from Thailand. This suggests that *R. mutabilis* is in *R.* sect. *Ingratae* Quél., but its placement in *R.* subsect. *Foetentinae* Melzer & Zvára or *R.* subsect. *Subvelatae* (Singer) Singer, represented by sequences labeled as *R. pulverulenta* Peck, cannot be resolved. *Russula pulverulenta* sequences were chosen because no sequences of *R. subvelata* Singer are available, and *R. pulverulenta* is a commonly collected species with the floccose pilear patches characteristic of *R.* subsect. *Subvelatae. Russula mutabilis* and *R. illota* share a benzaldehyde odor (characteristic of *R. fragrantissima* Romagn.), acrid taste, and a staining context, although Singer (1958), who considered its staining an artifact of the drying process, placed *R. mutabilis* in *R.* subsect. *Subvelatae* based on its velar remnants that showed a positive red reaction to potassium hydroxide. A similar species that exhibits the same extreme red staining on both the pileus and stipe is *R. ventricosipes* Peck, which may be a close relative separated by host preference (Adamčík et al. 2013).

The isotype of *R. westii* was recovered in a well-supported clade with members of *R.* subsect. *Subcompactinae* Singer, separate from a clade containing members of *R.* subsect. *Lilaceinae* Melzer & Zvára, including the type species. Morphological characters of *R. westii* are not inconsistent with placement in *R.* subsect. *Subcompactinae*, which includes a cream spore print, mild taste, and verrucose spores that lack an amyloid suprahilar spot (Murrill 1941). Singer (1958) noted an absence of pileocystidia and the presence of "none or few" primordial hyphae, but a preliminary morphological examination of the isotype by Adamčík revealed conspicuous pileocystidia. To determine whether the type collection was mixed and the TENN material represents a different species, the isotype was compared with a more detailed study of the FLAS holotype and confirmed as conspecific (Adamčík pers. comm.). Singer (1958) noted that *R. westii* was morphologically and ecologically very similar to *R. cremea* (Murrill) Singer and differed only in spore morphology.

Contrasting with Singer's (1986) classification, the phylogeny places the isotype of *R. australirosea* Murrill outside the clade representing *R.* subsect. *Lilaceinae,* united by the presence of primordial hyphae, a brightly colored pileus, and a pale spore print; unfortunately it is not possible at this time to place *R. australirosea* in another group. In his type study, Singer (1958) characterized *R. australirosea* with a completely mild taste and lacking dermatocystidia, characters consistent with *R.* subsect. *Lilaceinae* but described the spore print color as C to D, darker than expected for *R.* subsect. *Lilaceinae*. Singer (1958)

proposed that *R. australirosea* might be conspecific with *R. vinosirosea* Murrill, but we can reject this based on its phylogenetic placement.

Tree 2 (*Urentes* and *Decolorantes*) **Figure 1**

The *R. subrubescens* isotype and paratype form a clade with closely related sequences, but the sequences share only a 94% ITS similarity and are separated in two distinct clades with strong support. The BLAST results support the *R. subrubescens* type as closely related to *R. globispora* (J. Blum) Bon while placing the paratype in a clade with a sequence labeled "*R. dryadicola*" and another *R. globispora* sequence. Singer (1986) did not treat either of these taxa specifically, although he might have included them as varieties of *R. maculata* Quél., united by acrid taste, ochre spore print, absence of iodoform odor, and numerous dermatocystidia. Therefore, both entities should be included in *R.* subsect. *Urentes* Maire, not in *R.* sect. *Decolorantes* (Maire) Singer as represented by the type species *R. decolorans* (Fr.) Fr. Metadata associated with environmental sequences suggest that *R. subrubescens* is distributed throughout temperate North America as an associate of *Quercus*.

Tree 3 (*Russula*) **Figure 2**

Russula albimarginata Murrill and *R. emeticiformis* Murrill are both recovered in a clade with *R. vinacea* Burl., which is separate from the European representatives of *R. krombholzii* Shaffer[1] labeled "*R. atropurpurea*". Shaffer (1970) noted that *R. vinacea* reportedly has a less acrid taste and stronger yellowing flesh than *R. krombholzii*. The name *R. albimarginata* likely refers to the pallid margin described by Murrill (1945a) and observed in fresh collections (particularly in young fruitbodies) of *R. vinacea*. Singer's (1958) proposed placement of *R. emeticiformis* in *R.* subsect. *Russula* as a synonym of a nominal subspecies of *R. emetica* (Schaeff.) Pers. is not supported, as *R. emetica* is distantly related in *Russula* stirps Emetica. Singer's later (1975) placement of *R. emetica* subsp. *lacustris* Singer in *Russula* stirps Atropurpurea does, however, correspond with the current phylogenetic placement of *R. emeticiformis*. Both *R. albimarginata* and *R. emeticiformis* should be considered later taxonomic synonyms or closely related species to *R. vinacea*.

The isotype of *R. fragiloides* Murrill falls in a well-supported clade with members of *R.* subsect. *Russula* stirps Russula. Singer (1958) suggested that *R. fragiloides* is synonymous with *R. emetica* subsp. *emeticella* (Singer) Singer [≡ *R. emeticella* (Singer) Romagn.], but a recent revision by Hampe et al. (2013)

[1]Singer (1986: 824) used a heterotypic synonym, *R. bresadolae* Schulzer 1886, for the illegitimate later homonym *R. atropurpurea* (Krombh.) Britzelm. 1893, non Peck 1888. However the correct name for this taxon is *Russula krombholzii*, which was published as a replacement name (nom. nov.) based on the legitimate synonym *Agaricus atropurpureus* Krombh. 1845, and which therefore has priority over *R. bresadolae*.

found this taxon's position to be uncertain due to confusion over assigning a lectotype.

Tree 4 (*Xerampelinae* and Incertae sedis) **Figure 2**

Murrill (1945a,b) described *R. levyana* and *R. pinophila* Murrill with unchanging context, no odor, and — in the case of *R. pinophila* — a white gill color, features that would generally exclude these taxa from *R.* subsect. *Xerampelinae,* although he regarded *R. levyana* as related to *R. xerampelina* (Schaeff.) Fr. Adamčík & Buyck (2010), who evaluated the type and recent collections of *R. levyana,* noted these inconsistencies but explained that some American representatives of this group are known for having weak odors, especially in young fruitbodies. Younger fruitbodies might also explain gill whiteness, as *R.* subsect. *Xerampelinae* is characterized by ochraceous spore prints that make mature gills appear yellow. Phylogenetically, *R. levyana* and *R. pinophila* are in a well-supported clade with representatives of *R.* subsect. *Xerampelinae*. These two species share a 98% ITS sequence similarity and may represent the same species, a conclusion supported by their association with pine (Adamčík 2010). If so, *R. pinophila* should be regarded as a later taxonomic synonym of *R. levyana*. However, Buyck (pers. comm.) has suggested that species delimitation in this particular group should use a sequence similarity cutoff greater than 98%.

The isotype of *R. subcremeiceps* Murrill is closely related to *R. cremeoavellanea* Singer, which Singer (1986) placed in *R.* subsect. *Chamaeleontinae* Singer based on its eventual chocolate brown reaction to phenol, deep ochraceous spore print, and mild taste. Murrill (1946) did not test specimens for their phenol reaction, but described *R. subcremeiceps* as having a mild taste and pale yellow spore print. *Russula subcremeiceps* is somewhat allied with two other groups: *R.* subsect. *Vinosinae* Singer represented by *R. vinosa* Lindblad and *R.* subsect. *Integrae* Maire represented by *R. velenovskyi* Melzer & Zvára (Singer 1986). Both *R. cremeoavellanea* and *R. vinosa* are species that discolor, but Murrill (1946) described *R. subcremeiceps* as having unchanging flesh. He also noted that *R. subcremeiceps* closely resembled *R. albidicremea* Murrill but differed by having closer unforked gills and rounder spores. *Russula subcremeiceps* is a species widely distributed across eastern North America ranging from the northeast U.S. to Mexico.

Morphologically, *R. vinosirosea* is distinguished by its rose color with pale vinose tint and subequal stipe that is shorter than pileus width (Murrill 1943), two characters consistent with *R.* subsect. *Integrae* sensu Singer, of which *R. paludosa* Britzelm. is the type species. Although the isotype of *R. vinosirosea* shares 92% identity with *R. paludosa,* as well as *R. olivobrunnea* Ruots. & Vauras, *R. olivina* Ruots. & Vauras, and *R. veternosa* Fr., it cannot be confidently assigned to a taxonomic group due to low phylogenetic resolution.

Russula subsulphurea is distinguished by its large (9–10 cm) solitary habit, pallid to yellow-tinted to slightly rosy pileus, and white spore print (Murrill 1945b). The phylogenetic placement of the paratype specimen is unknown, but multiple environmental studies have frequently sampled *R. subsulphurea* or a closely related species in eastern North America.

The isotype of *R. brunneipes* Murrill is closely related to *R. umerensis* McNabb from New Zealand. These two species share many characters such as presence of dermatocystidia, vinaceous pileus color, a 7.5–10 µm spore size, and spores ornamented by mostly isolated echinulate 0.5–1 µm tall spines (Hesler 1960, McNabb 1973, Murrill 1945a). The *R. brunneipes* isotype shares a 92% identity with *R. cuprea* (Krombh.) J.E. Lange, *R. vinososordida* Ruots. & Vauras, and *R. vinosa*, but taxonomic placement is impossible at this time.

Tree 5 (*Amoeninae*) **Figure 3**

Russula alachuana Murrill and *R. variicolor* Murrill form a well-supported clade with other members of *R.* subsect. *Amoeninae* Singer. No reliable sequence of *R. amoena* Quél. was available, but all included taxa are united by having cheilocystidia without contents, no dermatocystidia, and a pale yellow to yellow spore print (Singer 1986). Both species form well-supported subclades separated from a well-sampled clade of specimens identified as *R. mariae* Peck, indicating that these are discrete phylogenetic species. Morphologically, *R. alachuana* is distinct by its large size, velvety pileus, and pulverulent stipe with rosy hue (Murrill 1938). An environmental sample of *R. alachuana* or closely related species has been sampled from Mexico. Murrill (1942) distinguished *R. variicolor,* potentially a trans-continental species sharing a close genetic affinity (99% similarity) to a *Pinus pinaster*-associated root tip sample from Europe, by its many-colored pileus that includes greenish hues and a completely white stipe.

Discussion

A molecular sequence of type material is an invaluable tool for modern systematics because it gives the taxonomist somewhat more objective criteria for naming phylogenetic groups and delimiting new species. The ITS region has been suggested as a barcoding region for ectomycorrhizal fungi due to the ease of its amplification and its ability to separate intraspecific from interspecific taxa (Schoch et al. 2012). My study illustrates a major hurdle in the revival of North American *Russula* taxonomy, which is that Murrill and his predecessors, G.S. Burlingham and C.H. Peck, described most *Russula* species in the 1910's–1940's. This means that North American type material is at the cusp of what can be successfully sequenced, and given the large number of species described and even more yet to be described, sequences of these taxa can greatly aid the revival of North American *Russula* systematics.

In this study I was able to produce ITS data from type collections designated by W.A. Murrill between 1938 and 1948 with a 66% success rate for collections accessioned at TENN. However, only the ITS1 region was successfully sequenced for a majority of Murrill's type material, and there are questions as to the value of using only ITS1 or ITS2 as a species barcode to separate closely related species. Either ITS region has been considered adequate for delimiting species in some groups of fungi, but this might not work as well in speciose groups, where species delimitation is performed at a fine scale with closely related taxa (Bengtsson-Palme et al. 2013).

Comparison of the isotype collection of *R. levyana* with FJ845433 (the specimen that shares a 99% sequence similarity) highlights the problem of using morphological or phylogenetic approaches separately in taxonomy. Either we must accept morphological variation in widespread species that may have been over-split and over-described or regard the ITS1 region as inadequate for separating closely related *Russula* species. To overcome these limitations, I suggest combining morphological recognition with molecular data to designate recently collected material as epitypes for historical North American *Russula* species. A good example can be found in *R. mutabilis*, where a partial sequence of the type shows a 100% sequence similarity with a recent collection from Texas that also agrees with the morphological diagnosis of the type.

Although partial sequence data may not allow for complete confidence in species delimitation, we are able to use such data to infer placement in clades, thereby supporting or refuting past classifications based on morphology. From the eight sampled taxa that have never before been placed in a group, I can now confidently place four: *R. albimarginata* in *R.* subsect. *Russula*, *R. pinophila* in *R.* subsect. *Xerampelinae*, *R. subcremeiceps* in *R.* subsect. *Chamaeleontinae*, and *R. subrubescens* in *R.* subsect. *Urentes* (Table 1). The results indicate a different or refined placement of *R. westii* in *R.* subsect. *Subcompactinae*, and we now have a more refined placement of *R. fragiloides*, *R. alachuana*, *R. variicolor,* and *R. emeticiformis* within their subsections (Table 1). Two taxa, *R. albimarginata* and *R. emeticiformis*, are proposed as possible synonyms with *R. vinacea*, and *R. pinophila* may be considered a possible synonym of *R. levyana*. Knowledge of group placement will aid in future systematic revisions, and those species not yet referable to an identifiable clade may be placed with additional molecular sampling and reliably identified sequences accessioned in GenBank and curated databases like UNITE.

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